



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/62, 15/13, C07K 15/28	A1	(11) International Publication Number: WO 93/15210 (43) International Publication Date: 5 August 1993 (05.08.93)
(21) International Application Number: PCT/EP93/00082 (22) International Filing Date: 15 January 1993 (15.01.93) (30) Priority data: 92101069.0 23 January 1992 (23.01.92) EP (34) Countries for which the regional or international application was filed: AT et al. (71) Applicant (for all designated States except US): MERCK PATENT GMBH [DE/DE]; Frankfurter Str. 250, Postbox 4119, D-6100 Darmstadt (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): PLÜCKTHUN, Andreas [DE/DE]; Jägerwirthstr. 3, D-8000 München 70 (DE). PACK, Peter [DE/DE]; Wilhelm-Busch-Str. 5/6, D-8000 München 71 (DE).		(81) Designated States: AU, CA, HU, JP, KR, NO, PL, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: MONOMERIC AND DIMERIC ANTIBODY-FRAGMENT FUSION PROTEINS (57) Abstract The present invention describes a new class of antigen binding molecules which contain Fv-fragments of an antibody but do not use the constant antibody domains. They can also dimerize with other antibody fragment molecules or with non-antibody fragment molecules to form bi- or multifunctional antibody-fragment fusion proteins and so-called miniantibodies, respectively. The new fusion proteins can be used in the broad field of diagnostic and therapeutical medicine.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TC	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

Monomeric and Dimeric Antibody-Fragment Fusion Proteins

5

The present invention describes a new class of antigen binding molecules which contain Fv-fragments of an antibody but do not use the constant antibody domains. They can also dimerize with other antibody fragment molecules or with non-antibody fragment molecules to form bi- or multifunctional antibody-fragment fusion proteins and so-called miniantibodies, respectively. The new fusion proteins can be used in the broad field of diagnostic and therapeutical medicine.

15

Background of the invention

Since a few years there is a great interest in the biotechnological field to modify naturally occurring antibodies in order to obtain more specified and more individual antibody species. Therefore, attempts have been made to produce (modified) antibody fragments.

20

All naturally occurring antibodies of all classes have at least two binding sites. This enables them to bind to a surface with a greater functional affinity (also called avidity) than monovalent fragments, such as Fab fragments.

25

Over the last few years, methods have been described (Skerra and Plückthun, 1988, Science 240, 1038-1040 ; Better et al., 1988, Science 240, 1041-1043) with which

30

2

functional antibody fragments can be produced in *Escherichia coli*. These include the Fv fragment (the heterodimer consisting of V_H and V_L) and the Fab fragment (consisting of the complete light chain with the domains V_L and C_L as well as the first two domains of the heavy chains V_H and C_{H1}).

The Fv fragment, however, has a tendency to dissociate into V_H and V_L and therefore, it is advantageous to link the two domains covalently. One particular way of linking them is by designing a peptide linker between them, either in the orientation V_H -linker- V_L or V_L -linker- V_H (Bird et al., 1988, Science 242, 423; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85, 5879) The resulting fragments are called single-chain Fv fragments.

All these fragments are, however, monovalent. We describe in this invention a method to engineer small dimerization domains based on peptides forming amphipathic helices. These peptides will be referred to as "intercalating", but this term is only meant to express the ability of targeted association and not a restriction referring to a particular structure of the dimerization interface.

While the methodology described here, is in principle applicable to either Fab, Fv or scFv fragments, it is the latter for which their use is most advantageous. In this case bivalent fragments can be constructed of very small size, and still the dissociation into V_L and V_H as well as the wrong matching of fragment chains, e.g. V_L - V_L , can be prevented.

Antibody fragments of small size are of particular advantage in many applications. In diagnostic applications (e. g. ELISA, RIA, etc.), the smaller molecules surface decreases the problems of nonspecific interactions, which are known to frequently involve the constant domains. The same is true in using antibody fragments as ligands in affinity chromatography. In tumor diagnostics or therapy, it is important that a significant proportion of the injected antibody penetrates tissues and localizes to the tumor, and is dependend on the molecular dimensions (Colcher et al., 1990, J. Natl. Cancer Inst. 82, 1191-1197). Expression yields and secretion efficiency of recombinant proteins are also a function of chain size (Skerra & Plückthun, 1991, Protein Eng. 4, 971) and smaller proteins are preferred for this reason. Therefore, molecules of a small size are advantageous for several reasons.

Previously, decreasing the molecular dimensions of the antibody meant the preparation of proteolytic fragments. The smallest bivalent fragments, (Fab)'₂ fragments, are still about twice the size of the present fragments of this invention. Therefore, these new fragments combine three features: (a) small size, (b) bivalence or bifunctionality and (c) ability of functional expression in E. coli.

There is great interest in bifunctional antibodies in a large number of areas. Bifunctional antibodies may be defined as having two different specificities for either two different antigens or for two epitopes of the same antigen.

There are currently a number of methods how to produce bifunctional antibodies. However, none of the existing methods allows to produce exclusively bifunctional antibodies in vivo, but rather a mixture of molecular species always occur, requiring complicated and expensive separation procedures.

Four principal methods can be distinguished. In the first, chemical crosslinking is used, which may advantageously use heterobifunctional crosslinkers. By this method, whole antibodies (Staerz et al., 1985, Nature 314, 628; Perez et al, 1985, Nature 316, 354-356), Fab fragments (Carter et al., 1992, Biotechnology 10, 163) and scFv fragments (Cumber et al., 1992, J. Immunol. 149, 120) have been chemically crosslinked after purification.

The second previous method involved the fusion of two hybridomas to give a so-called heterohybridoma or "quadroma". In this method, any light chain can pair with any heavy chain, and the two heavy chains can give homodimers or heterodimers resulting in very complicated product mixtures (Milstein & Cuello, 1983, Nature 305, 537).

The third method is related to the second and consists of transfecting two expression plasmids into a hybridoma cell, encoding the heavy and light chain of the second antibody (Lenz & Weidle, 1990, Gene 87, 213) or a retroviral vector (De Monte et al., 1990, Acad. Sci. 87, 2941-2945). However, once introduced, the product mixture is identical as in the second procedure.

30

Finally, antibodies have been reduced, mixed and reoxidized (Staerz & Bevan, 1986, Immunology Today 7). Again, very complicated product mixtures are obtained requiring sophisticated separation and quality control procedures.

5

Thus a method is still needed allowing the isolation of exclusively heterodimeric antibodies directly without the complicated preparation required from chemical crosslinking.

In the present invention, this problem is solved by (i)
10 covalently linking corresponding VH and VL domain in a scFv fragment and (ii) using dimerization domains only allowing the formation of heterodimers, such as certain leucine zip-pers and derivatives.

15 Another important consideration in the present invention was the desire to make the MW of the bispecific antibody as small as possible for reasons explained above in detail. This was achieved by using scFv fragments.

20 A number of uses of bispecific antibodies have been described, and most of them would benefit from this new technology. For example, bispecific antibodies are of great interest in tumor therapy. One arm of the antibody may bind to a tumor marker, the other arm to a T-cell epitope, a toxin, or a
25 radionuclide binding peptide or protein to bring a killing function close to the tumor cell. In diagnostics, one arm may bind to the analyte of interest and the other to a principle which can easily be quantified, e. g. an enzyme. Finally, in cellular applications, it may be advantageous to obtain
30 higher selectivity in binding, if two different epitopes or the same protein complex can be recognized or if two different proteins can be recognized on the same cell surface.

Thus, it was object of the invention to create new individual and stable antibody fragment fusion proteins with bi- or even multfunctional binding sites.

- 5 It has been found that antibody fragment fusion proteins containing Fv-fragments could be produced by genetic engineering methods which show specified and improved properties.

Object of the invention is, therefore, a monomeric antibody-
10 fragment fusion protein essentially consisting of a Fv-fragment of an antibody and a peptide which is capable to dimerize with another peptide by noncovalent interaction.

The term "noncovalent interaction" means every existing under
15 normal conditions stable linkage which is not related to a covalent binding, for example linkage by Van der Waal's forces, (steric) interdigitation of amphiphilic peptides, especially peptide helices, or peptides bearing opposite charges of amino acid residues. The correspondingly effective
20 peptides are called above and below interactive or intercalating peptides.

The amphiphilic peptides consist of up to 50 amino acids. Preferrably they consist of 10 to 30 amino acids. In a preferred
25 embodiment of the invention the interactive peptide is a peptide helix bundle (comprising of a helix, a turn and another helix, see above). In another embodiment the interactive peptide is a leucine zipper consisting of a peptide having several repeating amino acids, in which every seventh
30 amino acid is a leucine residue. In other cases according to the invention the peptide bear positively or negatively

charged residues, e.g. lysine (positively charged) or glutamic acid (negatively charged) in a way that this peptide can bind to another peptide (of a second monomeric unit) bearing opposite charges.

5

The Fv-fragment and the intercalating peptide are linked together either directly or by a linker peptide, preferably by a linker peptide. In a preferred embodiment the linker peptide is a hinge region sequence of an antibody.

10

As defined, the Fv-fragment consists of the V_L and V_H region of an antibody. The Fv-fragment according to the invention is preferably a single chain fragment. Single chain fragments can be obtained by standard techniques using standard linker molecules.

15

Furthermore, object of the invention is a dimeric fusion protein essentially consisting of two monomeric fusion proteins, wherein the linkage of the monomeric units bases on noncovalent interaction of identical or different peptides, characterized in that at least one monomeric unit is an antibody-Fv-fragment fusion protein as defined above.

20

If the dimer contains two Fv-fragments, the Fv-fragments may be the same (identical antigen binding sites) or may be different (different antigen binding sites). In these cases mono- and bispecific (Fv)- miniantibodies can be obtained. According to the invention bispecific mini-antibodies are preferred.

25

30

The interactive peptides may be the same or may be different; preferably, they are identical. The intercalating peptides may be associated in parallel or in antiparallel fashion.

5 Object of the invention is, therefore, above all, a dimeric fusion protein consisting of two Fv-fragments with different specificity (antigen binding sites) and identical intercalating helix peptides, the antibody fragments and the peptides are linked together by a hinge region sequence.

10

Furthermore, object of the invention is a dimer consisting of a monomeric unit containing a Fv-fragment and another monomeric unit wherein the Fv-Fragment was replaced by a non-antibody peptide. The non-antibody peptide may be a toxin, like
15 ricin, a chelator- or metal binding peptide, or an enzyme (e.g. marker enzyme), or a peptide bearing a detectable lable (e.g. a radioisotope).

The non-antibody peptide can also bear a corresponding binding site for said groups, binding sites directed to T-cells
20 or T-cell fragments included.

Furthermore, the invention relates to monomers and dimers, as defined above, wherein the interactive peptide(s) is (are)
25 additionally fused at the C-terminus to target proteins/peptides as mentioned above, the corresponding binding sites included. Thus, the resulting fusion proteins and miniantibodies, respectively, are multifunctional.

30

The invention relates, furthermore, to a process for preparation of a monomeric antibody fusion protein as defined above, characterized in that the genes coding for the Fv-fragment, the interactive peptide and, if desired, the linking peptide
5 are cloned into one expression plasmid, a host cell is transformed with said expression plasmid and cultivated in a nutrient solution, and the monomeric fusion protein is expressed in the cell or secreted into the medium.

10 Object of the invention is, finally, a process for preparation of a dimeric fusion protein as defined above, characterized in that the genes coding for the complete monomeric fusion proteins or parts of it are cloned at least into one expression plasmid, a host cell is transformed with said
15 expression plasmid(s) and cultivated in a nutrient solution, and either the complete dimeric fusion protein is expressed in the cell or into the medium, or the monomeric fusion proteins are separately expressed and the noncovalent linkage between the two monomeric units is performed in the
20 medium or in vitro, and in the case that only parts of the fusion proteins were cloned, protein engineering steps are additionally performed according to standard techniques.

The dimeric Fv-fragments containing fusion proteins according
25 to the invention show a high avidity against corresponding antigens and a satisfying stability. These novel bivalent or bifunctional molecules can be prepared as folded and assembled molecules in E. coli. These miniantibodies are compatible with functional expression by secretion.

Detailed description of the invention

The oligomerization domains were selected for having a fairly small molecular weight and for being compatible with transport of the fusion protein through the membrane. They are based on two different types of amphiphilic helices.

Amphiphilic helices are known to predominantly, but not exclusively, associate in two different molecular structures: Four helix bundles and coiled coils. The design and formation of helix bundles has been studied previously (Eisenberg et al., 1986, Proteins 1, 16-22; Ho and deGrado, 1987, J. Am. Chem. Soc. 109, 6751-6758; Regan and deGrado, 1988, Science 241, 976-978 ; Hill et al., 1990, Science 294, 543-546). This molecule association is also known from natural proteins (Richardson, 1981, Adv. Prot. Chem. 34, 167).

The four helix bundle may be formed from either four separate molecules (each contributing one helix), two molecules containing two helices each (connected as helix-turn-helix) or one molecule containing a helix-turn-helix-turn-helix-turn-helix motif. For dimerization or multimerization, only the first two are suitable.

Three variations of this latter theme were tested. In the first, one helix of the sequence given in Eisenberg et al. (1986) (Proteins 1, 16-22) was used. In the second, this sequence was extended by a small hydrophilic peptide ending in a cysteine. Once the helices are associated, the hydrophilic peptides are held in sufficiently close contact that they

can collide and a disulfide bond can form under oxidizing conditions, as in the periplasm of *E. coli*. In the third variation, two helices are used in tandem, separated by a short turn encoding peptide.

5

In the second design, peptides are used which can form so-called coiled-coil structures. Such peptides occur in transcription factors such as e. g. GCN4 from yeast and have been called leucine zippers (Landschulz et al., 1988, Science 240, 1759-1764). The crystal structure of this has been
10 solved recently (O'Shea et al., 1991, Science 254, 539-544) and showed a parallel arrangement of the helices.

A covalent attachment of the helices is possible by a small
15 peptide extension, again containing a cysteine. Since the helices are now parallel, the peptide extension can be much shorter, since the distance is much smaller.

The various dimerization devices (intercalating helices) were
20 however not fused to the antibody domain directly. It is advantageous to introduce a flexible peptide between the end of the scFv fragment and the beginning of the helix. As an example, the upper hinge region of mouse IgG3 has been used. However, a variety of hinges can be used. It is not required
25 for dimerization per se, but provides a spacing of the two scFv domains similar to the antigen binding sites of a whole antibody. This way, the two binding sites span a greater distance in space and therefore can reach neighboring antigens on a solid surface.

30

The naturally occurring hinges of antibodies are preferred embodiments of hinges in bivalent miniantibodies. In the case of bifunctional miniantibodies, the hinges may be shorter, since frequently molecules from different surfaces are to be crosslinked as close as possible, and flexibility of the dimer is not necessary. The choice of the hinge is governed by the desired residue sequence, length (Argos, 1990, J. Mol. Biol. 211, 943-958), compatibility with folding and stability of the amphiphilic helices (Richardson & Richardson, 1988, Science 240, 1648-1652), secretion and resistance against proteases .

The present invention deals with peptides as dimerization devices, which should be as small as possible. One preferred embodiment is the use of peptides which can form amphipathic helices. Such helices shield the hydrophobic surface by dimerization or even multimerization. Helices of this type are characterized by their having hydrophobic patches on one face of the helix, and containing a sufficient number of helix-forming residues. Rules for such peptides are discussed in Eisenberg et al. 1986, O'Shea et al., 1991 (Science 254, 539-544), 1992 (Cell 68, 699-708) .

Natural peptides of this type are found as the so-called leucine zippers, characterized by a periodic occurrence of leucine (every seventh residue) and other hydrophilic residues (e. g. valine) also every seventh residue. As these principles are now understood (O'Shea et al. 1991, 1992, literature cited), the sequence can be varied to incorporate

residues which make the association of homodimers unfavorable, but favors the association of heterodimers. Such sequence alteration can e. g. involve the incorporation of charge bridges, such that in the homodimers, like charges
5 repel each other and in the heterodimer, opposite charges attract each other (see below).

The present invention can also be extended to bifunctional miniantibodies. In this case, dimerization devices (inter-
10 calation peptides) have to be used which will only allow the formation of heterodimers, but not homodimers. A preferred embodiment of this part of the invention are two different coiled-coil helices, such as in naturally occurring leucine zippers, e. g. from the transcription factor proteins jun and
15 fos (O'Shea et al., 1989, Science 245, 646-648).

In a further embodiment of the invention, the constant scFv-hinge-helix can be extended at the C-terminus to result in a fusion protein. For example, a fusion to an enzyme may be
20 made to use such bivalent constructs in diagnostics. Such enzymes are e. g. alkaline phosphatase, luciferase or horse radish peroxidase. The advantage of such a antibody-enzyme fusion protein would be that the bivalence of the antibody would lead to an enhanced binding to the surface-bound anti-
25 gen. The advantage over a fusion protein prepared by conventional technology (i. e. chemical coupling of the antibody to the enzyme of choice) would be a greater batch-to-batch consistency; homogeneity of the product and the much simpler method of preparation, namely from E. coli in a single step.

In the same fashion, the miniantibodies may be extended at the C-terminus to incorporate a toxin. Such immunotoxins would be bivalent or even bispecific and thus combine the advantages of such antibody fragments linked above with the advantages in tumor therapy known for immunotoxins. Similarly, a metal binding peptide or protein could be linked genetically to be used in radioimmunotherapy or in tumor imaging. The same advantages for any genetically encoded hybrid protein hold true as given above for the antibody-enzyme fusions.

In another embodiment of the invention, a construct of the type scFv-hinge-helix may be made to dimerize with another protein fused to a dimerization domain, in complete analogy as described above for the formation of bispecific miniantibodies. In this fashion, the scFv fragment would e. g. be fitted with the helix of the fos protein. Such foreign protein, which could be made to form heterodimers with the scFv fragment, include enzymes useful in diagnosis, toxins, metal-binding peptides or proteins useful in radioimmunotherapy or radio-imaging.

Using the principles of this invention, the dimerization domains presented here can also serve for purification purposes. A recombinant protein of any kind can be fused to a dimerization domain, e.g. to hinge-fos-zipper. After coexpression with a scFv-hinge-jun, the heterodimer can be purified in one step with an affinity column for the scFv-specificity. In an alternative approach, the 'opposite' zipper, linked to a column support, 'catches' the protein-hinge-zipper when passing through the column as a crude cell extract.

The elution of the pure fusion protein from the column is possible using the unfolding temperature of the zipper. A subsequent separation from the dimerization domain is achievable by introduction of a proteolytic site, e.g. for blood clotting factor Xa, into the hinge (Nagai & Thogerson, 1987, Meth. Enzymol. 152,,461-481).

A particular advantage of the miniantibodies described in this invention is the ability to assemble functionally in *Escherichia coli*. In the case of homobivalent constructs, a dimerization principle is used which allows the formation of homodimers. Examples described above include the coiled-coil helix (leucine zipper) of the yeast protein GCN4 or the helices from an antiparallel 4-helix bundle. In this case, the scFv fragment is expressed in the presence of a bacterial signal sequence and carries at the end of the gene of the scFv fragment the codons for a hinge and the dimerization helix or helix-turn-helix. The helices are compatible with secretion to the periplasmic space in *E. coli*, where protein folding, disulfide formation and assembly occurs. Under these conditions, the homodimeric proteins form by themselves and can directly be isolated in the dimeric form.

If heterobivalent constructs are desired, two different scFv fragments or one scFv fragment associating with a different protein need to associate. In the preferred embodiment of this invention, both proteins to be assembled are expressed in the same cell, preferably on the same plasmid, preferably as a dicistronic operon. The design of artificial dicistronic

perons is explained e.g. in Skerra et al. (1991, Protein Eng. 4, 971). Since the assembly must take place in the periplasm, because the scFv fragment can only fold in the oxidizing milieu, both proteins must be transported and both must be fitted with a signal sequence. The dimerization peptides must be chosen such that they promote the association of two different proteins, but prevent the association of the respective homodimers. Examples of such proteins are the leucine zipper peptides of the proteins fos and jun (see above).

When not expressed in the same cell, the different scFv-hinge-zipper constructs have to be mixed together as a crude cell extract or purified protein and treated with raised temperature. In absence of the 'opposite' zipper, e.g. a scFv-hinge-jun-zipper construct is able to form homodimers. After short heating to the melting temperature of around 40°C, the zippers of the unwanted homodimer unfold and form a much more stable heterodimer (O'Shea et al., 1992, Cell 68, 699-708). Without raising the temperature, formation of heterodimers in vitro is not possible, as tested in experiments.

Short Description of the Figures and the Sequence Listing

Fig. 1 scFv-Expression vector pLISC-SE containing the scFv-fragment.

Fig. 2 Dicistronic scFv-hinge-zipper expression vector pACKxFyJ.

Fig. 3 Functional ELISA;

The concentrations of the affinity purified proteins, measured by OD₂₈₀ (vertical axis), refer to the molar number of binding sites per well (horizontal axis). The ELISA plates were coated with phosphocholine-BSA, and the purified phosphocholine-specific miniantibody-proteins were bound and detected by an anti-McPC603 antiserum.

(a) Comparison of various miniantibodies.

(b) Comparison of miniantibody scHLXc with ScFV and whole IgA.

Fig. 4 Functional Anti-lysozyme ELISA;

PC-affinity purified samples of coexpressed anti-PC-anti-lysozyme bispecific miniantibody. + and - on the horizontal axis means: plus inhibitor (+) and without inhibitor (-).

The attached sequence listing refers to sequence identity numbers (S.I.N.):

S.I.N. 1: Whole nucleotid- and amino acid sequence of the pLISC-SE vector.

S.I.N. 2: Gene cassette of intercalating GCN4-leucine zipper (nucleotid- and amino acid sequence).

S.I.N. 3: Gene cassette encoding intercalating antiparallel helix-turn-helix (nucleotid- and amino acid sequence).

- S.I.N. 4: Gene cassette encoding intercalating jun-zipper
and IgG3-hinge region.
- 5 S.I.N. 5: Gene cassette encoding intercalating fos-zipper
and IgG3-hinge region.
- S.I.N. 6: Gene cassette encoding intercalating jun-zipper
and designed linker.
- 10 S.I.N. 7: Gene cassette encoding intercalating fos-zipper
and designed linker.
- Example 1: Construction of vectors for secreted single-chain
fragments, containing a restriction site for
15 introducing genes for intercalating peptides.

Recombinant DNA-techniques were based on Sambrook et al.
(1989, Molecular Cloning: A laboratory manual. Second edi-
tion. Cold Spring Harbor Laboratory, New York). Functional
20 expression of the single-chain Fv fragments and the minian-
tibodies in E. coli JM83 was carried out with vectors
similar to pASK-lisc (Skerra et al., 1991, Protein Eng. 4,
971). Site directed mutagenesis was directly performed in
these vectors according to Kunkel et al. (1987, Meth. Enzy-
25 mol. 154, 367-382) and Geisselsoder et al. (1987, Biotechni-
ques 5, 786-791) using the helper phage M13K07 (Vieira &
Messing, 1987, Meth. Enzymol. 153, 3-11). SDS-PAGE was car-
ried out as described by Fling and Gregerson (1986, Anal.
Biochem. 155, 83-88). Concentrations of affinity-purified
30 proteins were measured by OD₂₈₀ using calculated extinction

coefficients (Gill & von Hippel, 1989, Anal. Biochem. 182, 319-326). A vector such as pASK40 (Skerra et al., 1991, Protein Eng. 4, 971) is used, which contains an origin of replication, a regulatable promotor, a bacterial signal sequence
5 followed by a multiple cloning site, a transcription terminator and an origin for single stranded phages. The gene for the single-chain Fv fragment is designed as follows: The nucleotide sequence of a V_H domain is directly followed by a linker sequence encoding preferably about 15 residues, preferably of
10 the sequence (Gly₄Ser)₃, followed directly by the sequence of the V_L domain. Alternatively, the sequence of the V_L domain may be directly followed by the sequence of the linker, followed by the sequence of the V_H domain.

15 If the antibody is of known sequence, the complete gene of the scFv fragment may be assembled from synthetic oligonucleotides. A detailed experimental procedure for such a gene synthesis of an antibody gene is e.g. given in Plückthun et al. (1987, Cold Spring Harbor Symp. Quant. Biol. 52,
20 105-112).

If the genes of the V_H and V_L domains are present in other vectors, the gene for the scFv fragment may be assembled from
25 restriction fragments. For example, a restriction fragment encoding most of the V_H domain may be excised from another plasmid, and a fragment encoding most of the V_L domain may be excised from a plasmid. The remaining pieces of V_L and V_H and

30

the linker for the scFv fragment can be provided by cassettes of synthetic oligonucleotides, which need to be ligated by standard methodology (Sambrook et al., 1989, literature cited). The mixture of fragments is ligated into the vector
5 pASK40 or a similar plasmid containing a pair of suitable restriction sites.

If the genes of the antibody have not been cloned before, they may be directly obtained from the hybridoma cell pro-
10 ducing the antibody by the polymerase chain reaction (PCR; PCR methodology is described in McPherson et al., 1991, PCR-A Practical Approach Oxford University Press, New York). Primers suitable for amplification of V_H and V_L domains have been given by Orlandi et al., 1989, Proc. Natl. Acad. Sci. USA 86,
15 3833-3837; Huse et al., 1989, Science 246, 1275-1281; Larrick et al., 1989, Bio-technology 7, 934-938. The methodology of obtaining mRNA from hybridoma is described in these references as well. The separate V_H and V_L genes may be cloned into separate vectors, and the scFv gene assembled according to
20 the principles explained above.

If the ligated fragments do not result in a correct reading frame of the scFv fragment, a precise fusion with the signal sequence codons resident on the plasmid may be generated by
25 site directed mutagenesis. The design of the oligonucleotides and the execution is possible for anyone skilled in the art.

5 The scFv expression plasmid so obtained contains the codons for a bacterial signal sequence, directly followed by the first variable domain (V_H or V_L), a linker and the second variable domain (V_L or V_H) under the control of a regulatable promoter.

10 At the 3' end of this genes, corresponding to the C-terminus of the scFv protein, a unique restriction site is introduced into the expression plasmid to allow insertion of cassettes coding for the intercalating peptides. The restriction site is introduced by site directed mutagenesis using the method of Kunkel (1987, Meth. Enzymol. 154, 367-382).

15 An example of the complete sequence of a suitable single-chain Fv expression plasmid pLISC-SE for receiving an intercalation peptide is shown in Fig. 1 and Sequence Identity No. (S.I.N.) 1 (see Sequence Listing).

20 **Example 2:** Design and construction of a gene cassette encoding intercalating peptides of a leucine zipper.

25 The gene cassette, fitted with restriction sites to be compatible with the restriction site at the 3' end of the scFv fragment gene, must encode the sequence of a hinge (connection the scFv fragment to the intercalating peptide) and the intercalation peptide itself. The hinge region, may however also be obmitted.

30 As an example the sequence of the upper hinge region of mouse IgG3 (Dangl et al., 1988, EMBO J. 7, 1989-1994), followed by the sequence of the leucine zipper sequence of the yeast protein GCN4 (Oas et al., 1990, Biochemistry T29, 2891-2894),

is back-translated into frequently used *E. coli* codons (S.I.N.: 2). Oligonucleotides are synthesized, and ligated into the vector pLISC-SE, previously digested with EcoRI and Hind III.

5

Example 3: Design and construction of a gene cassette encoding intercalating peptides of a four-helix bundle.

10 Analogous to Example 2, the sequence of the upper hinge region of mouse IgG3, followed by the sequence of the helix-turn-helix of a four helix bundle (Eisenberg et al., 1986, literature cited) is backtranslated into frequently used *E. coli* codons (S.I.N.: 3). Oligonucleotides are synthesized,
15 and ligated into the vector pLISC-SE, previously digested with Eco RI and Hind III.

Example 4: Design and construction two gene cassettes encoding intercalating peptides of a leucine zipper
20 and their co-expression.

Analogous to Example 2, the sequence of the upper hinge region of mouse IgG3 followed by the sequence of the zipper sequence of the jun protein (O'Shea et al., 1992, literature
25 cited), is backtranslated into frequently used *E. coli* codons (S.I.N.: 4). Oligonucleotides are synthesized, and ligated into the vector pLISC-SE, previously digested with EcoRI and Hind III.

30

In a parallel reaction, the sequence of the upper hinge region of mouse IgG3, followed by the sequence of the zipper sequence of the fos protein (O'Shea et al., 1992, Cell 68, 699-708), is backtranslated into frequently used E. coli codons (S.I.N.: 5). Oligonucleotides are synthesized, and
5 ligated into the vector pLISC-SE, previously digested with Eco RI and Hind III. The two vectors thus each code for a different antibody scFv fragment, followed by a hinge peptide and a different leucine zipper peptide. To co-express the two
10 scFv fragments, the whole scFv-hinge-zipper gene of the fos-containing product is excised from the vector as a Xba I-Hind III fragment and ligated into the vector, pLISC-SE-scFv-jun, containing already the scFv gene of the other antibody.
15 The newly obtained vector then expresses the scFv₁-linker₁-fos-zipper and the scFv₂-linker₂-jun-zipper from a single promoter as a dicistronic operon.

An improved sequence for the hinge region in the context of
20 fos and jun zippers is given in S.I.N.: 6 and 7. This hinge is shorter and therefore not as susceptible to proteolysis. In cases, where the distance between the two binding sites is of less importance, such shortened hinges may be advantageous. In this case, the "tail" of the scFv fragment has been
25 shortened and the EcoRI site, which receive the genes for the intercalating peptides has been moved four residues upstream.

Example 5: Purification of bivalent miniantibody from E. coli.

5 E. coli JM83, harboring a plasmid constructed as in examples II and III, are grown to an O.D. 550 of 0.5 and induced with IPTG at a final concentration of 1 mM. The cells are centrifuged, resuspended in BBS buffer (200 mM Na-borate, 160 mM NaCl, pH 8.0) and the suspension is passed through a French press. In these examples, a phosphorylcholine binding miniantibody is used. The miniantibody is purified by passage over
10 a phosphorylcholine affinity chromatography as described (Chesebro and Metzger, 1972, Biochemistry 11, 766-771).

Example 6: Purification of a bispecific miniantibody from E. coli
15

E. coli JM83, harboring a plasmid constructed as in examples II and III and containing a dicistronic structural gene for two different scFv (Fig. 2), are grown to an O.D. 550 of 0.5
20 and induced with IPTG at a final concentration of 1mM. The cells are centrifuged, resuspended in BBS buffer (200 mM Na-borate, 160 mM NaCl, pH 8.0) and the suspension is passed through a French press.

In this example, a bispecific miniantibody is used containing
25 both a specificity for phosphorylcholine as well as benzoyl-ampicillin. The miniantibody is purified by passage over a phosphorylcholine affinity chromatography as described (Chesebro and Metzger, 1972, literature cited).

Example 7: Surface binding of bivalent miniantibodies

The ELISA-plates (Nunc, Macrosorp) were coated with 400 µg/ml phosphocholine-BSA in PBS buffer (20 mM phosphate, pH 7.2, 115 mM NaCl). The hapten reagent was prepared from nitro-phenyl phosphocholine (Sigma), which was reduced and diazotized essentially as described (Chesebro & Metzger, 1972, literature cited), and reacted by azo-coupling to BSA (Sigma) in borate-saline buffer (52.5 mM sodium borate, pH 9, 120 mM NaCl) at 4°C for 48 hours with subsequent dialysis against PBS. After blocking the non-coated plate surface with 5% skim milk (Nestle) in PBS buffer for at least 2 hours, the periplasmic extract or the purified protein was incubated in BBS buffer on the plate for 90 min at room temperature. After thorough washing (3 times), remaining functional antibody fragments were detected according standard procedures (Harlow & Lane, 1988, "Antibodies, A Laboratory Manual", Cold Spring Harbor Laboratory, 555-592) with rabbit anti-McPC603 serum and anti-rabbit immunoglobulin linked to peroxidase (Sigma) according to Gallati (1979, Clin. Chem. Clin. Biochem. 17, 1-4).

An enormous gain in binding, and thus sensitivity, is observed for all miniantibody constructs, compared to the monomeric scFv fragment. This is consistent with the simultaneous binding of two or even more binding sites to the same surface. The avidity of the fusion protein SCHLXc was comparable to the natural antibody McPC603, which could be detected with antigen-coated ELISA, while the monomeric scFv fragment could only be detected with hundred-fold higher concentrations (Fig. 3 a, b). All binding is nearly totally

inhibitible with soluble hapten, except of the monomeric scFv fragment. The thermodynamic affinity of the natural antibody to soluble phosphocholine is about $1.6 \times 10^5 \text{ M}^{-1}$ and thus relatively weak (Metzger et al., 1971, Proceedings of the I
5 st Congress of Immunology. Academic Press, New York, pp. 253-267), and this is apparently not sufficient for a monomeric fragment-hapten complex to survive the repeated washing steps of a functional ELISA (Kemeny & Challacombe, 1988, "ELISA and other solid phase immunoassays", Wiley & Sons, New
10 York).

Example 8: Surface binding of bifunctional miniantibodies

Coexpressed bifunctional miniantibodies recognizing phosphorylcholine with one arm and lysozyme with the other arm
15 were purified by phosphocholine (PC) affinity chromatography and tested for lysozyme specificity. An ELISA-plate was coated with lysozyme, the ELISA was carried out as described in Example VII. Three different preparations show binding to
20 the antigen-surface, which is completely inhibitible with soluble lysozyme (Fig. 4).

25

30

SEQUENCE LISTING:

SEQ ID NO: 1

ACCCGACACC	ATCGAATGGC	GCAAAACCTT	TCGCGGTATG	GCATGATAGC	50
GCCCCGAAGA	GAGTCAATTC	AGGGTGGTGA	ATGTGAAACC	AGTAACGTTA	100
TACGATGTCG	CAGAGTATGC	CGGTGTCTCT	TATCAGACCG	TTTCCCGCGT	150
GGTGAACCAG	GCCAGCCACG	TTTCTGCGAA	AACGCGGGAA	AAAGTGGAAAG	200
CGGCGATGGC	GGAGCTGAAT	TACATTCCCA	ACCGCGTGGC	ACAACAACCTG	250
GCGGGCAAAC	AGTCGTTGCT	GATTGGCGTT	GCCACCTCCA	GTCTGGCCCT	300
GCACGCGCCG	TCGCAAATTG	TCGCGGCGAT	TAAATCTCGC	GCCGATCAAC	350
TGGGTGCCAG	CTGTGTGGTG	TCGATGGTAG	AACGAAGCGG	CGTCGAAGCC	400
TGTAAAGCGG	CGGTGCACAA	TCTTCTCGCG	CAACGCGTCA	GTGGGCTGAT	450
CATTAACTAT	CCGCTGGATG	ACCAGGATGC	CATTGCTGTG	GAAGCTGCCT	500
GCACTAATGT	TCCGGCGTTA	TTTCTTGATG	TCTCTGACCA	GACACCCATC	550
AACAGTATTA	TTTTCTCCCA	TGAAGACGGT	ACGCGACTGG	GCGTGAGCA	600
TCTGGTCGCA	TTGGGTCACC	AGCAAATCGC	GCTGTTAGCG	GGCCCATTAA	650
GTTCTGTCTC	GGCGCGTCTG	CGTCTGGCTG	GCTGGCATAA	ATATCTCACT	700
CGCAATCAAA	TTCAGCCGAT	AGCGGAACGG	GAAGGCGACT	GGAGTGCCAT	750
GTCCGGTFTT	CAACAAACCA	TGCAAATGCT	GAATGAGGGC	ATCGTTCCCA	800
CZGCGATGCT	GGTTGCCAAC	GATCAGATGG	CGCTGGGCGC	AATGCGCGCC	850
ATTACCGAGT	CCGGGCTGCG	CGTTGGTGCG	GATGTCTCGG	TAGTGGGATA	900
CGCAGATACC	GAAGACAGCT	CATGTTATAT	CCCGCCGTTA	ACCACCATCA	950
AACAGGATTT	TCGCCTGCTG	GGGCAAACCA	GCGTGGACCG	CTTGCTGCAA	1000
CTCTCTCAGG	GCCAGGCGGT	GAAGGGCAAT	CAGCTGTTGC	CCGTCTCACT	1050
GGTGAAGA	AAAACCACCC	TGGCGCCCAA	TACGCAAACC	GCCTCTCCCC	1100
GCGCGTTGGC	CGATTCAATTA	ATGCAGCTGG	CACGACAGGT	TTCCCGACTG	1150
GAAAGCGGGC	AGTGAGCGCA	ACGCAATTAA	TGTGAGTTAG	CTCACTCATT	1200
AGGCACCCCA	GGCTTTACAC	TTTATGCTTC	CGGCTCGTAT	GTTGTGTGGA	1250
ATTGTGAGCG	GATAACAATT	TCACACAGGA	AACAGCTATG	ACCATGATTA	1300
CGAATTTCTA	GATAACGAGG	GCAAAAAA	---ATG AAA AAG	ACA GCT ATC	1345
			Met Lys Lys	Thr Ala Ile	
			1	5	
GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG					1387
Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln					
	10	15	20		
GCC GAA GTT AAA CTG GTA GAG TCT GGT GGT GGT CTG GTA CAG					1429
Ala Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Gln					
	25	30			
CCG GGT GGA TCC CTG CGT CTG TCT TGC GCT ACC TCA GGT TTC					1471
Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Phe					
	35	40	45		
ACC TTC TCT GAC TTC TAC ATG GAG TGG GTA CGT CAG CCC CCG					1513
Thr Phe Ser Asp Phe Tyr Met Glu Trp Val Arg Gln Pro Pro					
	50	55	60		
GGT AAA CGT CTC GAG TGG ATC GCA GCT AGC CGT AAC AAA GGT					1555
Gly Lys Arg Leu Glu Trp Ile Ala Ala Ser Arg Asn Lys Gly					
	65	70	75		
AAC AAG TAT ACC ACC GAA TAC AGC GCT TCT GTT AAA GGT CGT					1597
Asn Lys Tyr Thr Thr Glu Tyr Ser Ala Ser Val Lys Gly Arg					
	80	85	90		

TTC	ATC	GTT	TCT	CGT	GAC	ACT	AGT	CAA	TCG	ATC	CTG	TAC	CTG	1639
Phe	Ile	Val	Ser	Arg	Asp	Thr	Ser	Gln	Ser	Ile	Leu	Tyr	Leu	
				95					100					
CAG	ATG	AAT	GCA	TTG	CGT	GCT	GAA	GAC	ACC	GCT	ATC	TAC	TAC	1681
Gln	Met	Asn	Ala	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	
105					110					115				
TGC	GCG	CGT	AAC	TAC	TAT	GGC	AGC	ACT	TGG	TAC	TTC	GAC	GTT	1723
Cys	Ala	Arg	Asn	Tyr	Tyr	Gly	Ser	Thr	Trp	Tyr	Phe	Asp	Val	
	120					125					130			
TGG	GGT	GCA	GGT	ACC	ACC	GTT	ACC	GTT	TCT	TCT	GGT	GGT	GGT	1765
Trp	Gly	Ala	Gly	Thr	Thr	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	
		135					140					145		
GGT	TCT	GGT	GGT	GGT	GGT	TCT	GGT	GGT	GGT	GGT	TCT	GAT	ATC	1807
Gly	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Asp	Ile	
			150					155					160	
GTT	ATG	ACC	CAG	TCT	CCG	AGC	TCT	CTG	TCT	GTA	TCT	GCA	GGT	1849
Val	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Val	Ser	Ala	Gly	
			165					170						
GAA	CGT	GTT	ACC	ATG	TCT	TGC	AAA	TCT	TCT	CAG	TCT	CTG	CTG	1891
Glu	Arg	Val	Thr	Met	Ser	Cys	Lys	Ser	Ser	Gln	Ser	Leu	Leu	
175					180					185				
AAC	TCT	GGT	AAC	CAG	AAA	AAC	TTC	CTG	GCG	TGG	TAT	CAG	CAA	1933
Asn	Ser	Gly	Asn	Gln	Lys	Asn	Phe	Leu	Ala	Trp	Tyr	Gln	Gln	
	190					195				200				
AAG	CCT	GGC	CAA	CCG	CCG	AAA	CTG	CTG	ATC	TAC	GGT	GCG	TCG	1975
Lsy	Pro	Gly	Gln	Pro	Pro	Lys	Leu	Leu	Ile	Tyr	Gly	Ala	Ser	
	205					210					215			
ACC	CGT	GAA	TCT	GGT	GTT	CCG	GAC	CGT	TTT	ACC	GGT	AGC	GGT	2017
Thr	Arg	Glu	Ser	Gly	Val	Pro	Asp	Arg	Phe	Thr	Gly	Ser	Gly	
		220						225				230		
AGC	GGT	ACC	GAC	TTC	ACT	CTG	ACC	ATC	TCT	TCT	GTA	CAG	GCT	2059
Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile	Ser	Ser	Val	Gln	Ala	
			235					240						
GAA	GAT	CTG	GCT	GTT	TAC	TAC	TGT	CAA	AAC	GAC	CAC	TCT	TAC	2101
Glu	Asp	Leu	Ala	Val	Tyr	Tyr	Cys	Gln	Asn	Asp	His	Ser	Tyr	
245				250					255					
CCG	CTG	ACC	TTT	GGC	GCC	GGC	ACC	AAA	CTG	GAA	CTG	AAG	CGC	2143
Pro	Leu	Thr	Phe	Gly	Ala	Gly	Thr	Lys	Leu	Glu	Leu	Lys	Arg	
	260					265					270			
GCT	AAC	GGT	GAA	TTC	TGATAAGCTTGA				CCTGTGAAGT		GAAAAATGGC			2190
Ala	Asn	Gly	Glu	Phe	*									
	275													
GCACATTGTG	CGACATTTTT				TTTGTCTGCC			GTTTACCGCT			ACTGCGTCAC			2240
GGATCCCCAC	GCGCCCTGTA				GCGGCGCATT			AAGCGCGGCG			GGTGTGGTGG			2290
TTACGCGCAG	CGTGACCGCT				ACACTTGCCA			GCGCCCTAGC			GCCCGCTCCT			2340
TTCGCTTTCT	TCCCTTCCTT				TCTCGCCACG			TTCGCCGGCT			TTCCCCGTCA			2390
AGCTCTAACT	CGGGGCACTT				CTTTAGGGTT			CCGATTTAGT			GCTTTACGGC			2440
ACCTCGAACC	GAAAAACTT				GATTAGGGTG			ATGGTTACAG			TAGTGGCCCA			2490
TCGCCCTGAT	AGACGGTTTT				TCGCCCTTGG			ACGTTGGAGT			CCACGTTCTT			2540
TAATAGTGGA	CTCTTGTTCC				AAACTGGAAC			AACACTCAAC			CCTATCTCGG			2590
TCTATTCTTT	TGATTTATAA				GGGATTTTGC			CGATTTCCGGC			CTATTGGTTA			2640

AAAAATGAGC	TGATTTAACA	AAAATTTAAC	GCGAATTTTA	ACAAAATATT	2690
AACGTTTACA	ATTTTCAGGTG	GCACTTTTCG	GGGAAATGTG	CGCGGAACCC	2740
CTATTTGTTT	ATTTTTCTAA	ATACATTCAA	ATATGTATCC	GCTCATGAGA	2790
CAATAACCCT	GATAAATGCT	TCAATAATAT	TGAAAAAGGA	AGAGTATGAG	2840
TATTCAACAT	TTCCGTGTCG	CCCTTATTCC	CTTTTTTGCG	GCATTTTGCC	2890
TTCCTGTTTT	TGCTCACCCA	GAAACGCTGG	TGAAAGTAAA	AGATGCTGAA	2940
GATCAGTTGG	GTGCACGAGT	GGGTACATC	GAACTGGATC	TCAACAGCGG	2990
TAAGATCCTT	GAGAGTTTTC	GCCCCGAAGA	ACGTTTTCCA	ATGATGAGCA	3040
CTTTTAAAGT	TCTGCTATGT	GGCGCGGTAT	TATCCCGTAT	TGACGCCGGG	3090
CAAGAGCAAC	TCCGTGCGCG	CATACACTAT	TCTCAGAATG	ACTTGGTGA	3140
GTACTCACCA	GTCACAGAAA	AGCATCTTAC	GGATGGCATG	ACAGTAAGAG	3190
AATTATGCAG	TGCTGCCATA	ACCATGAGTG	ATAACACTGC	GGCCAACTTA	3240
CTTCTGACAA	CGATCGGAGG	ACCGAAGGAG	CTAACCGCTT	TTTTGCACAA	3290
CATTGGGGAT	CATGTAACTC	GCCTTGATCG	TTGGGAACCG	GAGCTGAATG	3340
AAGCCATACC	AAACGACGAG	CGTGACACCA	CGATGCCTGT	AGCAATGGCA	3390
ACAACGTTGC	GCAAACTATT	AACTGGCGAA	CTACTTACTC	TAGCTTCCCG	3440
GCAACAATTA	ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	GGACCACTTC	3490
TGCGCTCGGC	CCTTCCGGCT	GGCTGGTTTA	TTGCTGATAA	ATCTGGAGCC	3540
GGTGAGCGTG	GGTCTCGCGG	TATCATTGCA	GCACTGGGGC	CAGATGGTAA	3590
GCCCTCCCGT	ATCGTAGTTA	TCTACACGAC	GGGGAGTCAG	GCAACTATGG	3640
ATGAACGAAA	TAGACAGATC	GCTGAGATAG	GTGCCTCACT	GATTAAAGCAT	3690
TGGTAACTGT	CAGACCAAGT	TTACTCATAT	ATACTTTAGA	TTGATTTAAA	3740
ACTTCATTTT	TAATTTAAAA	GGATCTAGGT	GAAGATCCTT	TTTGATAATC	3790
TCATGACCAA	AATCCCTTAA	CGTGAGTTTT	CGTTCCACTG	AGCGTCAGAC	3840
CCCGTAGAAA	AGATCAAAGG	ATCTTCTTGA	GATCCTTTTT	TTCTGCGCGT	3890
AATCTGCTGC	TTGCAAACAA	AAAAACCACC	GCTACCAGCG	GTGGTTTGTT	3940
TGCCGGATCA	AGAGCTACCA	ACTCTTTTTC	CGAAGGTAAC	TGGCTTCAGC	3990
AGAGCGCAGA	TACCAAATAC	TGTCCTTCTA	GTGTAGCCGT	AGTTAGGCCA	4040
CCACTTCAAG	AACTCTGTAG	CACCGCCTAC	ATACCTCGCT	CTGCTAATCC	4090
TGTTACCAGT	GGCTGCTGCC	AGTGGCGATA	AGTCGTGTCT	TACCGGGTTG	4140
GACTCAAGAC	GATAGTTACC	GGATAAGGCG	CAGCGGTCCG	GCTGAACGGG	4190
GGGTTCGTGC	ACACAGCCCA	GCTTGGAGCG	AACGACCTAC	ACCGAACTGA	4240
GATACCTACA	GCGTGAGCTA	TGAGAAAGCG	CCACGCTTCC	CGAAGGGAGA	4290
AAGGCGGACA	GGTATCCGGT	AAGCGGCAGG	GTCGGAACAG	GAGAGCGCAC	4340
GAGGGAGCTT	CCAGGGGGAA	ACGCCTGGTA	TCTTTATAGT	CCTGTCGGGT	4390
TTCGCCACCT	CTGACTTGAG	CGTCGATTTT	TGTGATGCTC	GTCAGGGGGG	4440
CGGAGCCTAT	GGAAAAACGC	CAGCAACGCG	GCCTTTTTTAC	GGTTCCTGGC	4490
CTTTTGCTGG	CCTTTTGCTC	ACATG			4515

SEQ ID NO: 2

```

GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CGC ATG 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Arg Met
1  ↑                    5                    10                    15
      L----- IgG3-hinge ----- L-----
AAA CAG CTG GAA GAT AAA GTT GAA GAG CTT CTT TCG AAA AAC TAC 90
Lys Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr
      20                    25                    30
----- GCN4-zipper -----
CAC CTC GAA AAT GAA GTT GCG CGC CTC AAA AAA CTT GTT GGT GAA 135
His Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu
      35                    40                    45
-----
CGC TGATAAGCTT GAC 151
Arg  ↑
---stop

```

SEQ ID NO: 3

```

GGT GAA TTC CCC AAA CCT AGC ACC CCC CCT GGC AGC AGT GGT GAA 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Gly Glu
1  ↑                    5                    10                    15
      L----- IgG3-hinge ----- L-----
CTG GAA GAG CTG CTT AAG CAT CTT AAA GAA CTT CTG AAG GGC CCC 90
Leu Glu Glu Leu Leu Lys His Leu Lys Glu Leu Leu Lys Gly Pro
      20                    25                    30
----- bundle-helix A ----- L-----
CGC AAA GGC GAA CTC GAG GAA CTG CTG AAA CAT CTG AAG GAG CTG 135
Arg Lys Gly Glu Leu Glu Glu Leu Leu Lys His Leu Lys Glu Leu
      35                    40                    45
-turn- L----- bundle-helix B -----
CTT AAA GGT GAA TTC TGATAAGCTT GACCTGTGAA GTGAAAAAAT G 191
Leu Lys Gly Glu Phe
      ↑ 50 ↑
-----stop

```


SEQ ID NO: 4

```

GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CGT ATC 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Arg Ile
1  ↑                    5                    10                    15
      L-----IgG3-hinge-----J L-----
GCT CGT CTC GAG GAA AAA GTT AAA ACC CTG AAA GCT CAG AAC TCC 90
Ala Arg Leu Glu Glu Lys Val Lys Thr Leu Lys Ala Gln Asn Ser
                20                    25                    30
----- jun-zipper -----
GAA CTG GCT TCC ACC GCT AAC ATG CTG CGT GAA CAG GTT GCT CAG 135
Glu Leu Ala Ser Thr Ala Asn Met Leu Arg Glu Gln Val Ala Gln
                35                    40                    45
----- jun-zipper -----
CTG AAA CAG AAA GTT ATG AAC TAC TGATAAGCTT GACCTGTGAA G 180
Leu Lys Gln Lys Val Met Asn Tyr
                50
-----J stop

```

SEQ ID NO: 5

```

GGT GAA TTC CCC AAA CCT AGT ACT CCC CCT GGC AGC AGC CTG ACC 45
Gly Glu Phe Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Leu Thr
1  ↑                    5                    10                    15
      L-----IgG3-hinge-----J L-----
GAC ACC CTG CAG GCT GAA ACC GAC CAG CTG GAA GAC AAA AAA TCC 90
Asp Thr Leu Gln Ala Glu Thr Asp Gln Leu Glu Asp Lys Lys Ser
                20                    25                    30
----- fos-zipper -----
GCT CTG CAG ACC GAA ATC GCT AAC CTG CTG AAA GAA AAA GAA AAA 135
Ala Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys
                35                    40                    45
----- fos-zipper -----
CTG GAA TTT ATC CTG GCT GCT TAC TGATAAGCTT GACCTGTGAA G 180
Leu Glu Phe Ile Leu Ala Ala Tyr
                50
-----J stop

```

SEQ ID NO: 6

```

GGT GAA TTC CCG TCT GGT AAC GAA GCT CGT ATC GCT CGT CTC GAG 45
Gly Glu Phe Pro Ser Gly Asn Glu Ala Arg Ile Ala Arg Leu Glu
1  ↑                    5                10                15
      L----- linker -----J L-----
GAA AAA GTT AAA ACC CTG AAA GCT CAG AAC TCC GAA CTG GCT TCC 90
Glu Lys Val Lys Thr Leu Lys Ala Gln Asn Ser Glu Leu Ala Ser
      20                25                30
----- jun-zipper -----
ACC GCT AAC ATG CTG CGT GAA CAG GTT GCT CAG CTG AAA CAG AAA 135
Thr Ala Asn Met Leu Arg Glu Gln Val Ala Gln Leu Lys Gln Lys
      35                40                45
----- jun-zipper -----
GTT ATG AAC TAC TGATAAGCTT GACCTGTGAA GTGAAAAATG GCG 180
Val Met Asn Tyr      ↑
-----J stop

```

SEQ ID NO: 7

```

GGT GAA TTC GGT CCG TCT GGT AAC GAA CTG ACC GAC ACC CTG CAG 45
Gly Glu Phe Gly Pro Ser Gly Asn Glu Leu Thr Asp Thr Leu Gln
1  ↑                    5                10                15
      L----- linker -----J L----- fos-zipper -----
GCT GAA ACC GAC CAG CTG GAA GAC AAA AAA TCC GCT CTG CAG ACC 90
Ala Glu Thr Asp Gln Leu Glu Asp Lys Lys Ser Ala Leu Gln Thr
      20                25                30
----- fos-zipper -----
GAA ATC GCT AAC CTG CTG AAA GAA AAA GAA AAA CTG GAA TTT ATC 135
Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile
      35                40                45
----- fos-zipper -----
CTG GCT GCT TAC TGATAAGCTT GACCTGTGAA GTGAAAAATGGCG 180
Leu Ala Ala Tyr      ↑
-----J stop

```

5

Patent claims

1. Monomeric antibody-fragment fusion protein essentially
consisting of a Fv-fragment of an antibody and a peptide
10 which is capable to dimerize with another peptide by
noncovalent interaction.
2. Monomer according to claim 1 characterized in that the
Fv-fragment is a single chain fragment.
- 15 3. Monomer according to claim 1 or 2 characterized in that
the interactive peptide consists of 10 to 50, preferably
10 to 30 amino acids.
- 20 4. Monomer according to one of the claims 1 or 3 characteri-
zed in that the peptide consists of at least one helix.
5. Monomer according to claim 4 characterized in that the
helix peptide consists of a helix, a turn and another
25 helix.
6. Monomer according to claims 4 characterized in that the
peptide contains a leucine zipper molecule, having
several repeating amino acids, in which every seventh
30 amino acid is a leucine.

7. Monomer according to claims 4 characterized in that the peptide bears charged residues.
8. Monomer according one of the claims 1 to 7 characterized in that a linking peptide is between the Fv-fragment and the peptide.
9. Monomer according to claim 8 characterized in that the linking peptide is a hinge region sequence of an antibody or a fragment thereof.
10. Process for preparation of a monomeric antibody fusion protein as defined in claims 1 to 9, characterized in that the genes coding for the Fv-fragment, the interactive peptide and, if desired, the linking peptide are cloned into one expression plasmid, a host cell is transformed with said expression plasmid and cultivated in a nutrient solution, and the monomeric fusion protein is expressed in the cell or secreted into the medium.
11. Process according to claim 10 characterized in that the host cell is E. coli.
12. Dimeric fusion protein essentially consisting of two monomeric fusion proteins, wherein the linkage of the monomeric units bases on noncovalent interaction of identical or different peptides, characterized in that at least one monomeric unit is an antibody-fragment fusion protein as defined in claims 1 to 9.

13. Dimeric fusion protein according to claim 12 wherein the interactive peptides are the same.
- 5 14. Dimer according to claim 12 or 13 characterized in that the second monomeric unit is an antibody-fragment fusion protein as defined in claims 1 to 9 having different specificity.
- 10 15. Dimer according to claim 12 or 13 characterized in that the second monomeric unit is a fusion protein as defined in claims 1 to 9, wherein the antibody-fragment (Fv) is replaced by a non-antibody protein or peptide.
- 15 16. Dimer according to claim 15 characterized in that the protein or peptide is a toxin, a chelator peptide, a metal binding protein or an enzyme, or has the corresponding specific binding site.
- 20 17. Dimer according to claim 15 characterized in that the protein or peptide has a T-cell-, or a T-cell fragment specific binding site.
- 25 18. Dimer according to one of the claims 12 to 17, wherein another protein is fused at the C-terminus of one or both of the intercalating peptides.
- 30 19. Dimer according to claim 18, wherein the fused protein is a toxin, a chelator peptide, a metal binding protein or an enzyme, or has the corresponding specific binding site, or has a T-cell (fragment) specific binding site.

20. Process for preparation of a dimeric fusion proteins defined in claims 12 to 19 characterized in that the genes coding for the complete monomeric fusion proteins or parts of it are cloned at least into one expression
5 plasmid, a host cell is transformed with said expression plasmid(s) and cultivated in a nutrient solution, and either the complete dimeric fusion protein is expressed in the cell or into the medium, or the monomeric fusion proteins are separately expressed and the noncovalent
10 linkage between the two monomeric units is performed in the medium or in vitro, and in the case that only parts of the fusion proteins were cloned protein engineering steps are additionally performed.
- 15 21. Process according to claim 20 characterized in that the gene coding for the first monomeric fusion protein is cloned into a first expression plasmid, and the gene coding for the second monomeric fusion protein is cloned into a second expression plasmid.
- 20 22. Process according to claim 20 characterized in that the noncovalent linkage between the monomeric units forming the dimeric fusion protein is performed in vitro.

25

30

23. Process according to one of the claims 20 to 22 characterized in that the host cell is *E. coli*.

5 24. Construction kit for preparation of selective dimers of antibody-fragment fusion proteins as defined in claims 12 to 19 containing (a) a monomeric antibody-fragment fusion protein as defined in claims 1 to 8, and (b) a second monomeric fusion protein as defined in (a), wherein the antibody fragment has the same or another antigen specificity, or wherein the antibody fragment unit is replaced
10 by a non-antibody protein/peptide.

15

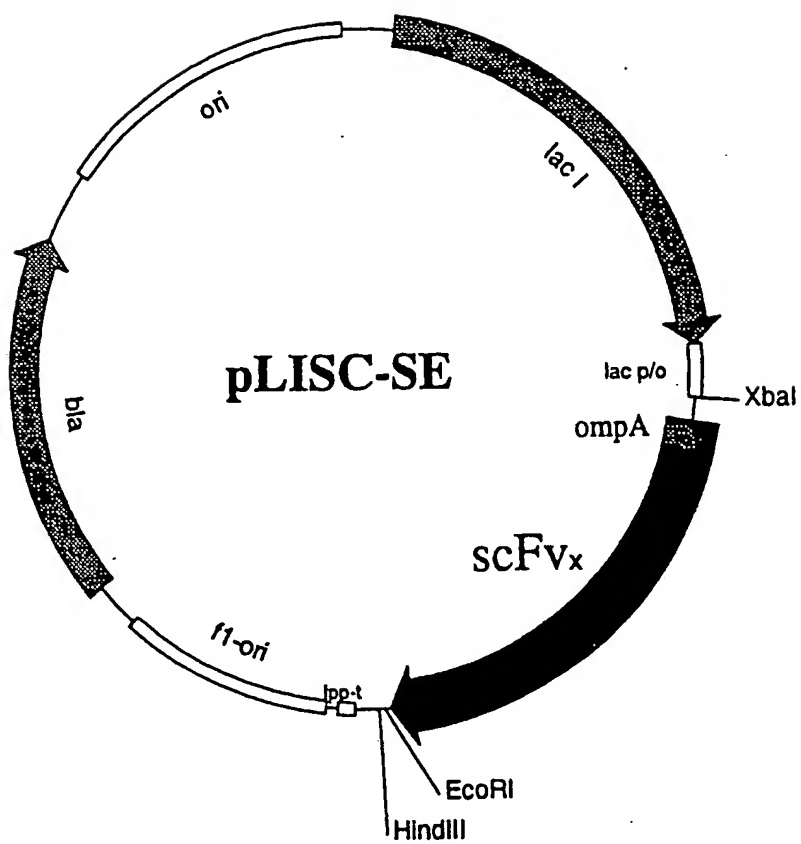
20

25

30

1 / 4

FIG. 1



2 / 4

Fig. 2

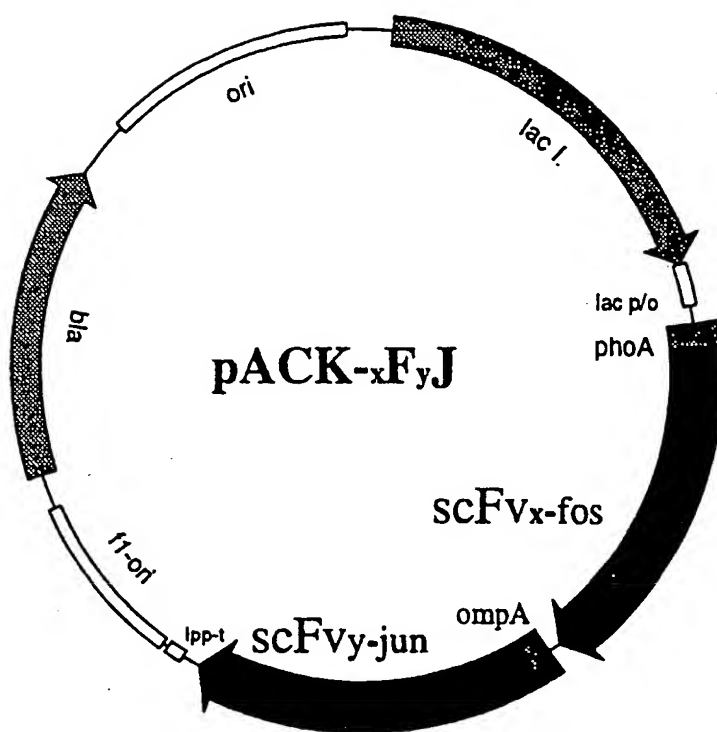


Fig. 3 a

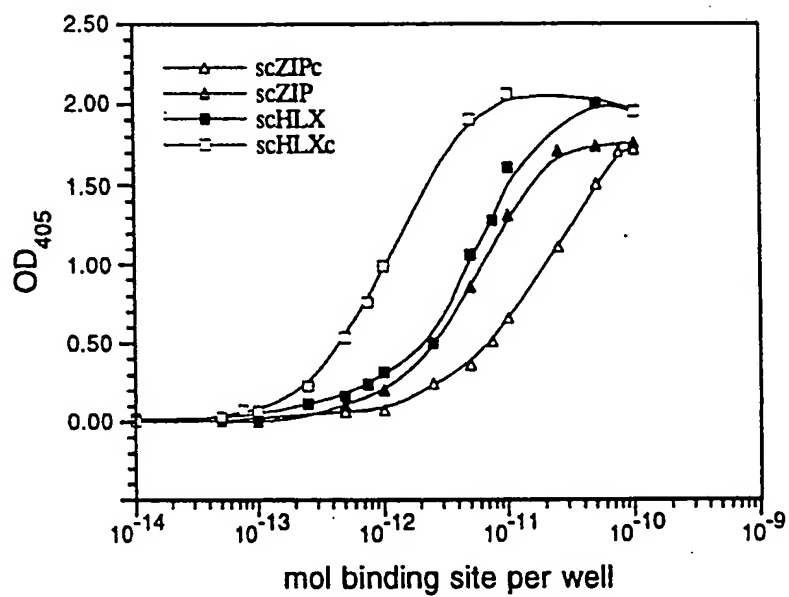


Fig. 3 b

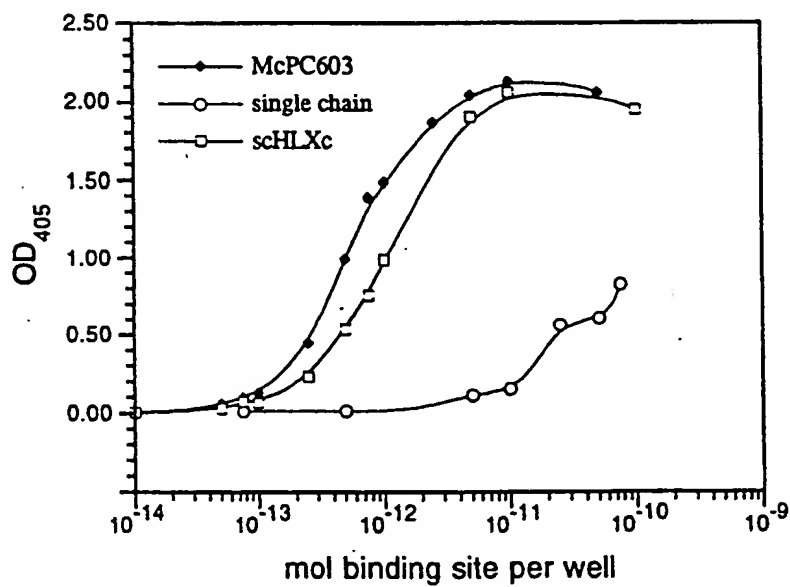
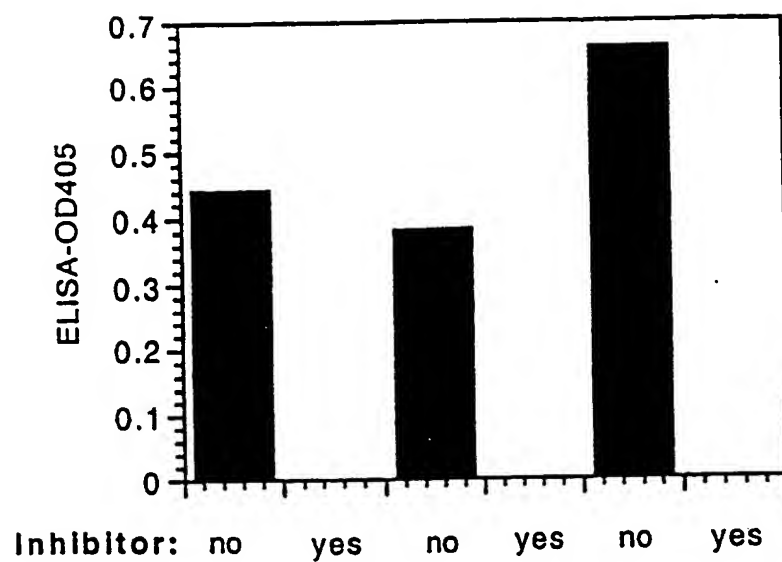


Fig. 4



INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 93/00082

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/62; C12N15/13; C07K15/28		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	TRENDS IN BIOTECHNOLOGY vol. 9, April 1991, CAMBRIDGE GB pages 132 - 137 BIRD AND WALKER 'Single chain antibody variable regions' see page 136, column 3, paragraph 2 ---	1-7, 10-24
Y	PROTEIN ENGINEERING vol. 4, no. 4, April 1991, ENGLAND GB pages 457 - 461 BLONDEL AND BEDOUELLE 'Engineering the quaternary structure of an exported protein with a leucine zipper' see page 461, column 1, last paragraph --- -/--	1-7, 10-24
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 01 JUNE 1993		Date of Mailing of this International Search Report 21 -06- 1993
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer CUPIDO M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document ¹ , with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	<p>BIOCHEMISTRY vol. 31, no. 6, 18 February 1992, EASTON, PA US pages 1579 - 1584 PACK AND PLUCKTHUN 'Miniantibodies: Use of amphipatic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in Escherichia coli' see the whole document -----</p>	1-24